Effect of Fatty Acid Conjugation on Antimicrobial Peptide Activity

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ABSTRACT

Two peptides, YGAA[KKAAKAA]$_2$ (AKK), and YG[AKAKAAKA]$_2$ (KAK) were conjugated to fatty acids and tested for the effect on their structure, antibacterial activity, and eukaryotic cell toxicity. In the presence of bacteria-mimicking phospholipid vesicles fatty acid conjugates of the amphipathic peptide, AKK, show a larger change in helical structure than either of the unmodified peptides or the conjugate of the non-amphipathic peptide, KAK. The induction of helicity corresponds to a significant improvement in antimicrobial activity as measured by a minimum bactericidal concentration test. Lengthening the fatty acid conjugate improves the antimicrobial activity of AKK as long as the peptide has less than 16 carbons. Peptide AKK conjugated to fatty acids with more than 16 carbons show no antimicrobial activity. Investigation of peptide and peptide amphiphile structure in the presence of red blood cell-mimicking phospholipid vesicles shows that neither the peptides, nor the fatty acid conjugated peptides have any significant helical structure. Neither the peptides nor their lauric acid conjugates showed significant hemolytic activity.

INTRODUCTION

Antimicrobial peptides are part of the innate immunity of multi-cellular organisms (Zasloff, M., 2002). Antimicrobial peptides are diverse in sequence and structure, but share two common features; a net positive charge which allows them to preferentially target the negatively-charged cell membranes of prokaryotes such as bacteria, and the ability to assume a three dimensional structure with distinct hydrophobic and hydrophilic faces, called an amphipathic structure. A common class of antimicrobial peptide has the feature that they adopt an amphipathic α-helical structure in the presence of membranes but are unfolded in solution (Hancock, R. E. W. and Rozek, A., 2002; Zhang, L. J., Rozek, A., and Hancock, R. E. W., 2001). The formation of an amphipathic structure seems to be required for membrane binding and lytic activity (Blondelle, S. E. and Houghten, R. A., 1992; Giangaspero, A., Sandri, L., and Tossi, A., 2001). When the concentration of peptide bound to the membrane reaches a critical concentration, the peptides either insert into the membrane to form pores, or destabilize the membrane by disrupting the membrane curvature (Shai, Y., 2002), either of which could be fatal to a cell.

The novel killing mechanism of antimicrobial peptides makes them an interesting alternative to traditional antibiotics, as target bacteria may be less able to develop resistances. However, for these peptides to be effective as systemic therapeutics their antimicrobial activity must be increased without raising their toxicity to eukaryotic cells (Darveau, R. P., Cunningham, M. D., Seachord, C. L., Cassianoclough, L., Cosand, W. L., Blake, J., and Watkins, C. S., 1991).

Some antimicrobial peptides, such as polymyxin (Benedict, R. G. and Langlykke, A. F., 1947) and lipopetaibols (Toniolo, C., Crisma, M., Formaggio, F., Peggion, C., Epand, R. F., and Epand, R. M., 2001) are naturally coupled to fatty acid tails. Removal of the fatty acid tail decreases the antimicrobial activity of polymyxin (Tsubery, H., Ofek, I., Cohen, S., and Fridkin, M., 2001) demonstrating that it is important to peptide activity. This phenomenon has been used to raise bactericidal activity of other antimicrobial peptides by conjugating them to fatty acids(Avrahami, D. and Shai, Y., 2002; Majerle, A., Kidric, J., and Jerala, R., 2001; Wakabayashi, H., Matsumoto, H., Hashimoto, K., Teraguchi, S., Takase, M., and Hayasawa, H., 1999).

To study the effect of fatty acid tail addition on peptide secondary structure, as well as the correlation between peptide secondary structure and antimicrobial activity, we have conjugated two peptides to various fatty acids (**Figure 1**). Peptide AKK is based on the sequence of a previously studied synthetic leucine-lysine antimicrobial peptide that segregates its hydrophobic and hydrophilic amino acids into distinct faces on a helix (Haynie, S. L., Crum, G. A., and Doele, B. A., 1995). Peptide KAK is designed not to form amphipathic helices but contains the same amino acid sequence as

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Figure 1: **A) Fatty acid conjugated peptide structure. The region in the brackets is the peptide region. B) Helical Wheel Diagram of peptides KAK (left) and AKK (right). The black filled circles are charged amino acids.**

peptide AKK (Marqusee, S., Robbins, V. H., and Baldwin, R. L., 1989). The peptides were tested for their antimicrobial and hemolytic activity. Additionally the aggregation state and peptide was structure was studied.

MATERIALS AND METHODS

Peptide amphiphile is prepared using resin bound peptide received from Synpep Corporation. Resin-bound peptide was deprotected with 20 vol% piperidine in dimethyl formaldehyde (DMF) for 2 hours. Following deprotection the resin-bound peptide was washed three times with DMF, then coupled to either lauric acid, myristic acid, palmitic acid or arachidic acid using a four-fold molar excess of N-hydrobenzotriazole, 2-(1H-Benzotriazole-1yl)1,1,3,3-tetramethyluronium hexafluorophosphate, N,N'-diisopropylethylamine, and lauric acid.

AKK, KAK, C12-AKK, C14-AKK, C16-AKK, C20-AKK, and C12-KAK were cleaved using a mixture of 95/5 vol% trifloroacetic acid (TFA)/water solution and precipitated in cold methyl-tert-butyl-ether. Peptides were purified by reverse phase HPLC on a Vydac C4 column.

Phospholipid vesicles were prepared by dissolving either 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), or 1,2-dilauroyl-sn-glycero-3-phospoethanolamine (DLPE) and 1,2-dilauroyl-sn-glycero-3-phospoglycerol (DLPG) in a 70/30 wt% DLPE/DLPG ratio, in chloroform. The solution was then dried under nitrogen to form a thin film. The film was then further dried under vacuum for at least 12 h. The lipids were dispersed in 10 mM sodium phosphate buffer, pH 7.4, to a concentration of 2.4 mM and extruded through a 100 nm Millipore polycarbonate filter 5 times.

Circular dichroism spectra were recorded at 37 ^{o}C in 10 mM sodium phosphate buffer, pH 7.4, with a peptide concentration of 0.02 mM. Spectra were also recorded in the presence of either DLPE/DLPG or DLPC vesicles at a vesicle concentration of 0.3 mM. To estimate secondary structure contribution, a least squares fit was preformed using a linear combination of standard α-helix and random coil spectra (Reed, J. and Reed, T. A., 1997).

The aggregation state of the peptide amphiphiles was determined by measurement of surface tension. Surface tension was measured using a Wilhemly plate. Peptide is added to a solution of 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and allowed to equilibrate before the surface tension was measured.

The peptides were tested against two strains of Gram-negative *Escherichia coli* (DH5α and ML-35) or Gram-positive *S. epidermidis* (ATCC#12228). Mid-logarithmic phase bacteria cultures were diluted in Tryptic soy broth (TSB) to concentrations of $1x10^5$ – $1x10^6$ colony forming units (CFU)/mL. Test peptide was added to the bacteria solution at varying concentrations and the mixture incubated for 18 h at 37 ^{o}C. Following incubation 50 μL aliquots were plated out and grown overnight. The MBC was taken as the lowest concentration where no colonies grew.

To determine the extent of hemolysis fresh human red blood cells were centrifuged and washed with phosphate buffered saline (PBS). The red blood cells were diluted to a concentration of 0.4 vol% in PBS. Peptide or peptide amphiphile was added at various concentrations and the samples were incubated for 1 h at 37 ^{o}C. The samples were centrifuged to sediment the intact red blood cells and UV absorbance at 414 nm was used to determine hemolytic activity. 100% hemolysis was determined by addition of 1 vol% Triton-X100. The percentage of hemolysis was calculated as follows:

% hemolysis= 100 (A_{414} with peptide - A_{414} in plain PBS)/(A_{414} in Triton-X100 – A_{414} in plain PBS)

RESULTS AND DISCUSSION

Peptide AKK was conjugated to lauric acid, myristric acid, palmitic acid, or arachidic acid (named C12-AKK, C14-AKK, C16-AKK, C20-AKK respectively). Peptide KAK was conjugated to lauric acid (named C12-KAK).

Bactericidal activities of peptides AKK, KAK, and the peptide amphiphiles C12-AKK, C14-AKK, C16-AKK, C20-AKK, and C12-KAK, were measured against two strains of the Gram-negative bacteria *E. coli* (ML-35 and DH5α) and one strain of the Gram-positive bacteria *S. epidermidis* (ATCC#12228) (**Figure 2**). MBC values above 65 are considered to be not antimicrobial. Neither of the unmodified peptides had an MBC below 65 µM. C12-AKK was moderately antimicrobial. C14-AKK showed greater activity than C12-AKK against all bacteria studied. C16-AKK showed the highest activity against the gram negative bacteria, but had no activity towards the gram positive *S. epidermidis*. Neither C12-KAK nor C20-AKK showed bactericidal activity in this assay.

The toxicities of the peptides and lauric acid conjugates of the peptides towards eukaryotic cells were tested by their ability to lyse human red blood cells. AKK, C12-AKK, KAK and C12-KAK show no
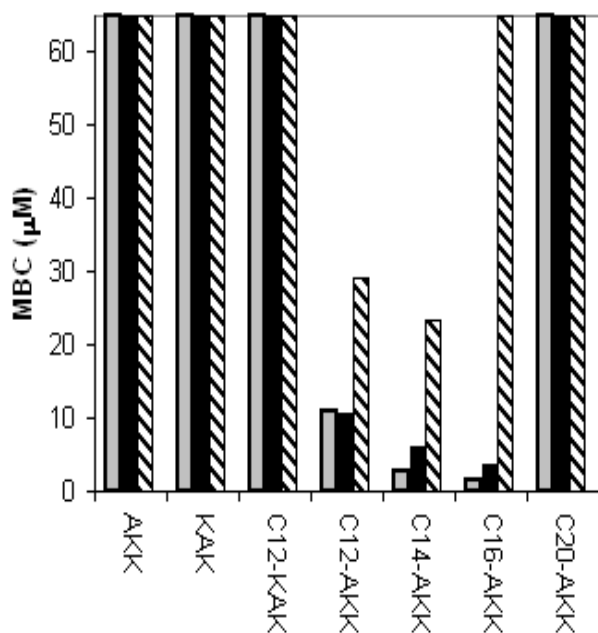
Figure 2: MBC of peptides and peptide amphilies against *E. coli* DH5α (grey), *E. coli* ML-35 (black), and *S. epidermidis* (striped).
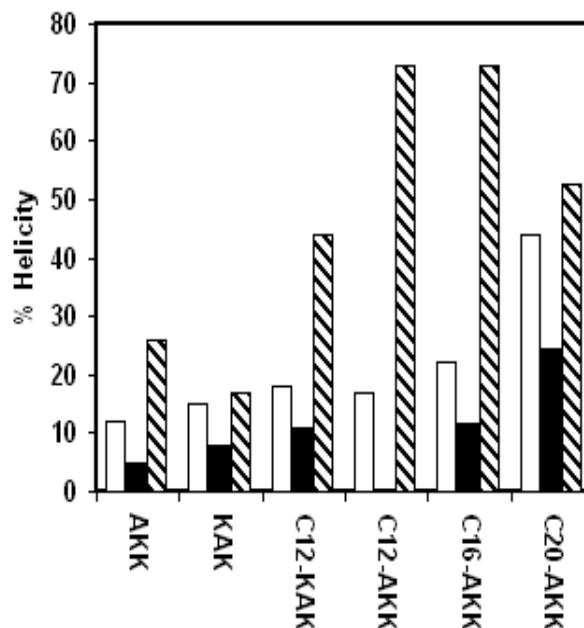
Figure 3: Amount of helicity of peptides and peptide amphiphiles in NaPO$_4$ (white), with PC vesicles (black), or with PE/PG vesicles (striped)

detectable hemolytic activity up to 60 µM and mild (<15%) hemolysis at 500 µM (data not shown).

Circular dichroism was used to assess the structure of the peptide and peptide amphiphiles in various environments (data not shown). The resulting spectra were fit to evaluate the helical content in each environment (**Figure 3**). In benign sodium phosphate buffer only the C20-AKK peptide showed significant helical content (<25%). In the presence of red blood cell mimicking zwitterionic DLPC vesicles all the peptides and peptide conjugates showed a decrease in helical structure as compared to the buffer samples. When the peptides were added to DLPE/DLPG vesicles, a mimic for bacteria outer membranes, C12-AKK and C16-AKK shows a large degree of helical structure, much more than the unmodified AKK peptide. C12-KAK also shows a larger degree of helix formation than the KAK peptide, but the change is not as significant as the C12-AKK or C16-AKK peptides. C20-AKK also shows an increase in helical structure, the change is also small.

The CMC of the C12-AKK, C14-AKK and C16-AKK peptide amphiphiles was measured. The CMC of C12-AKK is above 1mM. The CMC of C14-AKK and C16-AKK to within a 7% error are 220 and 16mM respectively.

Since amphipathicity is requisite for antimicrobial action KAK is not expected to be a good antimicrobial agent. Although the addition of the fatty acid tail increases the hydrophobicity of KAK, it does not show a
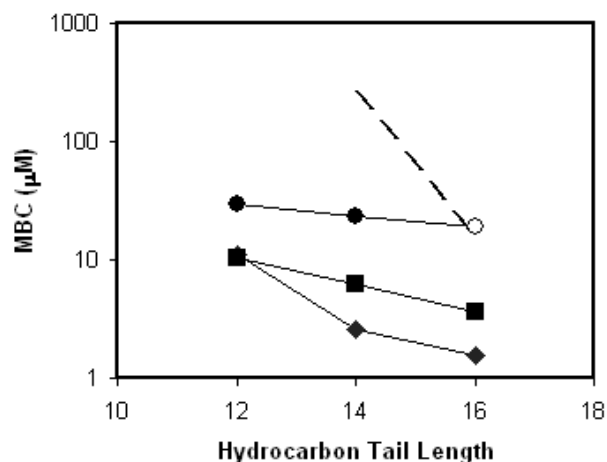
Figure 4: CMC of AKK as a function of tail length (dashed line). MBC of AKK as a function of tail length for peptide amphiphiles against *E. coli* DH5α (diamonds), *E. coli* ML-35 (squares), and *S. epidermidis* (filled circles). The projected MBC of C16-AKK against *S. epdpermidis* is shown as an open circle.

detectable increase in antimicrobial activity as measured by the MBC test. Studies show that some non-amphipathic peptides have mild bactericidal activity against Gram-negative bacteria (Tossi, A., Sandri, L., and Giangaspero, A., 2000). It is possible that such peptides fold into structures other than α-helices in order to maximize their amphipathicity and allow them to be mildly antimicrobial(Oren, Z., Lerman, J. C., Gudmundsson, G. H., Agerberth, B., and Shai, Y., 1999). This may also be the case with C12-KAK; we speculate that it may form a helical structure with either greater or fewer amino acids per turn than an α-helix.

AKK is highly amphipathic. However, it lacks strongly hydrophobic amino acids thus the unmodified peptide shows no antimicrobial activity. Conjugation of the peptide to a fatty acid increases its affinity to cell membranes. When the peptide is bound to a bacterial cell membrane it can fold and kill the cell, thus increasing the peptides affinity to cell membranes should increase its activity. This seems to be the case with the C12-AKK and C14-AKK peptide amphiphiles towards all bacteria strains tested. The lack of activity of C16-AKK towards *S. epidermidis* and C20-AKK against all bacteria tested is due to self-assembly of the peptide in solution at the concentration requisite for activity. Figure 3 shows the MBC and CMC of peptide AKK as a function of tail length. We see that the peptide is active

as long as the MBC is below the CMC. The MBC of C16-AKK against *S. epidermidis* is projected to be 20 μM, higher than the CMC of C16-AKK. The observation that antimicrobial activity is limited by CMC is consistent with results obtained for simple antimicrobial surfactants(Birnie, C. R., Malamud, D., and Schnaare, R. L., 2000; Ross, S., Kwartler, C. E., and Bailey, J. H., 1953; Tomlinson, E., Brown, M. R. W., and Davis, S. S., 1977).

Interestingly none of the peptides or their lauric acid conjugates show significant hemolytic activity. This is consistent with the fact that the peptides and peptide amphiphiles do no fold in the presence of PC vesicles.

CONCLUSION

Our results indicate conjugation of fatty acids to antimicrobial peptides facilitates the interaction with bacterial cell membranes leading to increased antimicrobial activity. Studies into the structure of the peptide in the presence of bacteria membrane-mimicking phospholipids show a greater degree of structuring of the modified peptides, implying a greater interaction with the membrane. The length of the fatty acid tail is important since lengthening the tail increases the activity of the peptide. However, lengthening the tail also decreases the CMC of the peptide amphiphile. If the CMC of the peptide amphiphile is too low it will lose its antimicrobial activity. It is interesting that the improvement of bactericidal activity for C12-AKK is not accompanied by an increase in hemolytic activity. The addition of fatty acid tails to form peptide amphiphiles may be a viable strategy to improve the antimicrobial activity of peptides.

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